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Developmental Biology 270 (2004) 525–537

DEVELOPMENTAL
BIOLOGYwww.elsevier.com/locate/ydbio

Genomes & Developmental Control

A conserved enhancer element that drives FGF4 gene expression in the embryonic myotomes is synergistically activated by GATA and bHLH proteins

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Received for publication 31 October 2003, revised 10 February 2004, accepted 11 March 2004

Available online 9 April 2004

Abstract

FGF4 is the earliest member of the fibroblast growth factor (FGF) family expressed during embryogenesis where it plays essential roles in post-implantation development and limb growth and patterning. The expression of the *Fgf4* gene in specific developmental stages, including the ICM of the blastocyst, the myotomes, and the limb bud AER, is regulated by distinct enhancer elements (*Hom*) in the 3' UTR. We previously identified the *Hom3a* region as the major DNA element responsible for *Fgf4* expression in the myotomes and AER, and showed that a conserved E-box is a target for the myogenic bHLH transcription factors MYF5 and MYOD. To further define the *cis*- and *trans*-acting elements that determine *Hom3a* activity, we conducted a mutational analysis of the ability of the *Hom3a* region to drive *lacZ* expression in the myotomes of transgenic mice. We identified a minimal enhancer of 226nt that contains four elements, including the E-box, necessary to drive gene expression in the myotomes. One of these elements is a binding site for the GATA family of transcription factors, and we show here that GATA 1–4 and 6 can synergize with MYF5 or MYOD to activate transcription of a reporter plasmid driven by a portion of the *Hom3a* enhancer including the GATA site and the E-box. In line with this finding, we could show a direct interaction between MYF5/MYOD and GATA-3 or GATA-4, mediated by the N-terminal and bHLH domains of MYF5/MYOD and the C-terminal zinc finger domain of GATA-3/4. To further study the role of the *Hom3a* enhancer in directing *Fgf4* expression and the function of FGF4 in limb and muscle development, we generated mutant mice in which the *Fgf4 Hom3a* region had been deleted ($\Delta 3a$). In situ hybridization analysis of sections from $\Delta 3a/\Delta 3a$ embryos at E11.5 showed a drastically reduced expression of *Fgf4* mRNA in the myotomes and AER. However, these mice developed normally and show no limb or muscle defects, and the same was true of heterozygous mice in which one *Fgf4* allele carried the *Hom3a* deletion and the other was a null allele ($\Delta 3a/Fgf4^{-/-}$). Together, these results show that *Hom3a* is the major DNA enhancer element directing *Fgf4* expression in myotomes and limb bud AER, and that its activity in the myotomes results at least in part from the synergistic action of GATA and bHLH myogenic factors that bind to evolutionary conserved sequences in the *Hom3a* enhancer. However, expression of *Fgf4* in the myotomes or AER of murine embryos does not appear to be essential for muscle or limb development.

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Keywords: FGF4; GATA; bHLH; MYOD; MYF5; Myotome; Myogenesis

Introduction

Fibroblast growth factors (FGFs) form a large family of signaling molecules involved in early embryonic development and organogenesis (Basilico and Moscatelli, 1992; Goldfarb, 1996; Ornitz and Itoh, 2001). Among the FGFs,

FGF4 is the earliest expressed FGF during embryogenesis and has been shown to play an essential role in post-implantation mouse development, and a less essential, but important role in limb growth and patterning (Feldman et al., 1995; Sun et al., 2002). FGF4 transcripts are first detected in the ICM of the blastocyst at E4.5 and in embryonal stem cells, and then in the primitive streak (E7), branchial arches and myotomes (E9.5–13.5), the apical ectodermal ridge (AER) of limb buds (E10.5–11.5) and the tooth buds (E14.5) (Drucker and Goldfarb, 1993; Niswander and Martin, 1992). At later stages of develop-

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ment, the expression of *Fgf4* is no longer detected, remaining silent in adult.

The mechanisms regulating expression of *Fgf4* during embryonic development have been the object of numerous studies. We originally showed that expression of *Fgf4* in EC and ES cells, and thus presumably in the ICM of the blastocyst, is determined by an enhancer element in the 3'UTR of the *Fgf4* gene and that this enhancer element is the target of the transcriptional regulators SOX2 and OCT-3, which cooperatively bind to adjacent sequences in the EC cell enhancer to synergistically activate transcription (Ambrosetti et al., 1997; Dailey and Basilico, 2001; Yuan et al., 1995). That SOX2 and OCT-3 are the major transactivators of *Fgf4* in the ICM have been confirmed by in vivo transgenic studies and by studies of *Sox2* and *Oct-3* knockout embryos (Avilion et al., 2002; Fraidenraich et al., 1998; Nichols et al., 1998). The extension of these studies to other stages of development showed that expression of *Fgf4* in myotomes, limb bud AER, and tooth bud is also regulated by 3' UTR enhancer elements that are distinct from those driving *Fgf4* expression in the blastocyst (Fraidenraich et al., 1998). The *Fgf4* 3' UTR contains three conserved DNA blocks, *Hom 1*, *2*, and *3* (subdivided into *3a* and *3b*). *Hom3b* corresponds to the previously mentioned EC cell enhancer. Using a transgenic approach, we established that the *Hom3a* region contains overlapping but distinct DNA elements capable of driving *lacZ* expression in the myotomes and AER of mouse embryos. Additional elements contained within the *Hom2* region were found to contribute to *Fgf4* expression in the rostral myotomes (Fraidenraich et al., 1998, 2000). Interestingly, the same *Hom2* region was recently reported to direct *Fgf4* expression in the tooth bud (Kratochwil et al., 2002).

We also showed that the *Hom3a* element contains a conserved E-box, and that this E-box is a target of myogenic bHLH factors MYF5 and MYOD (Fraidenraich et al., 2000). The analysis of *Myf5* and *MyoD* knockout mice harboring *Fgf4-lacZ* transgenes indicated that MYF5 is the physiological transactivator of *Fgf4* transcription in the myotomes, but that MYOD could support expression of *Fgf4* in the ventral myotomes in the absence of MYF5 (Fraidenraich et al., 2000). However, the expression of *Fgf4* in these structures must be regulated by additional factors, since MYF5 and MYOD expression in the myotomes is much more widespread than that of FGF4, that is restricted to a central portion of the myotomes, and many tissues and cell lines express MYF5 or MYOD but not FGF4 (Bober et al., 1991; Braun et al., 1992, 1994; Fraidenraich et al., 2000).

In order to identify the *cis*- and *trans*-acting elements which govern the myotomal expression of *Fgf4*, we have now further dissected the functional elements within the *Hom3a* region using mutational analysis. We show that three additional regions, which comprise a putative GATA factor binding site (Ko and Engel, 1993; Merika and Orkin, 1993), a putative binding site for the family of transcription enhancer factors (TEFs) (Jacquemin et al., 1996; Kaneko

and DePamphilis, 1998) and a sequence of unknown function, are required besides the E-box.

The GATA family of transcription factors includes six members that have been shown to play critical roles in regulating tissue-specific gene expression (Charron and Nemer, 1999; Molkentin, 2000). We show that GATA factors bind to a cognate site in the *Hom3a* enhancer, and that they can synergize with MYF5 or MYOD to activate transcription of a reporter plasmid driven by a portion of the *Fgf4 Hom3a* enhancer. GATA-3 and -4 also interact with MYF5 and MYOD in the absence of DNA.

Since *Fgf4* null embryos do not develop past the implantation stage (Feldman et al., 1995), the effect of FGF4 ablation at later stages of development has been difficult to study. Thus, to further probe the role of the *Hom3a* enhancer element in directing *Fgf4* expression in the myotomes and limb bud AER and the function of FGF4 in limb and muscle development, we generated mice in which the *Fgf4 Hom3a* region had been deleted. These mice developed beyond the implantation stage and expressed very reduced amounts of *Fgf4* transcripts in the myotomes and AER, confirming that *Hom3a* is the major myotomal/AER *Fgf4* enhancer element. However, these mice display no limb or muscle phenotype, indicating that *Fgf4* expression in these embryonic structures is not essential for their further development.

Materials and methods

Plasmid DNAs

The pGNA-*lacZ* vector (Fraidenraich et al., 1998) was used for the transient transgenesis experiments. The following *Fgf4* 3' UTR regions were inserted downstream to the *lacZ* gene, respectively; 3a Control (6251–6600), 3a1 (6251–6428), 3a2(6429–6600), 3a3(6251–6505), 3a4 (6336–6600), 3a5(6336–6505), 3a6(6280–6505), 3a7 (6308–6505). The nucleotides in the following 13 regions of *Fgf4* 3'UTR were substitutionally mutated keeping the length of nt 6251–6600, and inserted in the same site of the *lacZ* construct; 3aMEF2(6508–6517), Mut1 (6291–6300), Mut2 (6306–6317), Mut3 (6323–6332), Mut4 (6338–6349), Mut5 (6367–6378), Mut6 (6384–6395), Mut7 (6401–6412), Mut8 (6418–6430), Mut9 (6436–6447), Mut10 (6453–6464), Mut11 (6470–6481), Mut12 (6487–6498). All the constructs derived from *Fgf4* 3'UTR were made by PCR amplification.

cDNA of *MyoD* and *Myf5* in the vector pCMVhygro were gifts from M. Buckingham. For pull-down assay, cDNAs of *MyoD* and *Myf5* were amplified by PCR and inserted to *Bam*HI–*Eco*RI site of pGEX-2T, respectively (Amersham Pharmacia Biotech). Mutated *MyoD* and *Myf5* cDNAs were also made by PCR and inserted to the pGEX-2T in the same way.

GATA cDNAs were kind gifts from these researchers: GATA-1 and GATA-2, M. Yamamoto (University of Tsu-

kuba, Japan); GATA-3, D. Engel (University of Michigan), GATA-4, E. N. Olson (The University of Texas Southwestern Medical Center); GATA-5 and GATA-6, S. Musco (University of Pennsylvania) and R. Patient (University of Nottingham, UK). GATA cDNAs were amplified by PCR and inserted into pcDNA 3.1(–)/Myc-HisA vector (Invitrogen) and used for transient transfection to cells. For pull-down assay, cDNAs of GATA-3 and GATA-4 and their mutants were amplified by PCR and inserted downstream to T7 polymerase binding site of pcDNA1 (Invitrogen).

To make the *Fgf4*-CAT constructs, *Fgf4*-CAT plasmid derived from the pCAT3-Basic Vector (Promega) containing the minimal murine *Fgf4* promoter (nt 64 to nt 150) was used (Ambrosetti et al., 2000). To generate constructs pHom3a4xCAT (nt 6292–6305/6339–6366) oligonucleotides containing the GATA binding site, TEF binding site and E-box2 of *Hom3a* were multimerized and inserted upstream to the *Fgf4* promoter. The enhancers which have a mutated GATA binding site and/or a mutated E-box2 were made in the same way.

Transient transgenesis experiments

Standard microinjection of linearized DNA into the pronucleus of fertilized eggs was performed at the NYU Transgenic Mouse Facility using Swiss Webster or FVB/N mouse. X-gal staining of whole embryo was performed at E11.5 as described previously (Fraidenraich et al., 1998). Incorporation of the transgene into the embryos was tested on yolk sac DNA. At least five transgenic embryos were examined for X-gal staining to define the construct as *lacZ*-negative.

Transfection and CAT assay

Calcium phosphate transfections were performed as described previously (Curatola and Basilico, 1990). HeLa cells were transfected with 2 µg of DNA containing various reporter plasmid; pHom3a4xCAT, no enhancer-CAT, GATA mut-CAT, E-box mut-CAT, and GATA E-box mut-CAT with or without 0.1 µg of expression vectors; pcDNA 3.1(–)/Myc-HisA-GATA-1, -2, -3, -4, -5, -6, and pCMVhygro-*MyoD*, -*Myf5*. CMV-β-galactosidase (1 µg) was used as internal control. CAT activity was measured as described previously (Curatola and Basilico, 1990).

Electrophoretic mobility shift assay

Whole cell extracts (WCE) were prepared from transfected HeLa cells as previously described (Fraidenraich et al., 2000). Oligonucleotides were labeled with [γ -³²P] ATP and T4 polynucleotide kinase. Oligonucleotides used in electrophoretic mobility shift assay are WT (5'-CACGTTCCAGCTATCTTTACCCAATG-3') and MUT (5'-GCTTCTCTCCACATGTAGTATCTGG-3'). Binding reactions contained 6 µg of whole cell extract, 40 mM Hepes, 4% Ficoll, 40 mM KCl, 0.05% NP40, 75 ng of poly

(dI-dC) and approximately 30,000 cpm ³²P-labeled probe and 200-fold molar excess of competitor oligonucleotides where appropriate. Protein binding was allowed to proceed for 30 min at room temperature. The samples were immediately loaded onto 4% polyacrylamide gels containing 0.5xTBE and ran at 20 mA at room temperature for 1.5 h.

Immunoblotting

Two hundred ninety-three cells were transfected by pcDNA 3.1(–)/Myc-HisA-GATA-1, -2, -3, -4, -5, and -6, respectively. WCE were prepared and loaded onto SDS-PAGE and blotted into nitrocellulose membranes (OSMONICS). Immunoblotting was performed using the mAb 9E10 anti-human c-myc antibody (Roche) at 1:1000 dilution. The primary antibody was detected using anti-mouse horseradish-peroxidase conjugated secondary antibody (Promega) in TBS containing 0.4% Tween20 and ECL reagent as described by the manufacturer (Amersham Pharmacia).

GST pull-down assay

All the GST fusion proteins were expressed in *Escherichia coli* BL21 and purified by GST sepharose (Amersham Pharmacia). Binding assay was performed with labeled protein synthesized in vitro using rabbit reticulocyte lysate system or wheat germ extract system (Promega) in the presence of ³⁵S-labeled methionine (Perkin Elmer) according to the manufacturer's protocol. Equal amounts of immobilized GST fusion proteins were incubated overnight at 4°C with 10 µl of ³⁵S-labeled proteins in GST-binding buffer containing 40 mM Hepes pH 7.2, 50 mM Na acetate pH7.0, 200 mM NaCl, 2 mM EDTA, 5 mM dithiothreitol, 0.5% Nonidet P-40, protease inhibitors, and 2 µg of bovine serum albumin/ml. After five washes in GST binding buffer, beads were boiled in SDS sample buffer to elute bound protein, which was subsequently resolved by SDS-PAGE and analyzed by autoradiography.

Generation of *Hom3a* knockout mice

To generate *Fgf4 Hom3a* knockout mice, two fragments of *Fgf4* (5' arm; 3.7 kb-*SphI*–*EcoRI* fragment; nt 2446–6150, 3' arm; 1.8 kb-*VspI*–*EcoNI* fragment; nt 6509–8300) were inserted into 5' and 3' of the *neo^r* cassette of pKSloxPNT (a gift from A. L. Joyner). The linearized plasmid was electroporated into W4 embryonic stem cells. Transfected cells were selected by G418 as previously described (Ortega et al., 1998). Three G418 resistant homologous recombinant clones were injected into C57BL/6 blastocysts. The chimeras were crossed with CMV-*Cre* females to remove *neo^r* gene. The heterozygote pups ($\Delta 3a/+$) were crossed with *Fgf4* heterozygotes (*Fgf4*^{+/–}) to generate $\Delta 3a/Fgf4$ [–] mice. Heterozygous *Fgf4* knockout mice (*Fgf4*^{+/–}) were provided by V.E. Papaioannou.

In situ hybridization

The murine *Fgf4* 3' UTR (410 nt, including *Hom2* region) and coding region (620 nt) were used to generate ^{33}P -labeled RNA probes with T3 and T7 RNA polymerases, 12 μM cold UTP and 4 μM ^{33}P -UTP. The hybridization procedure was performed essentially as previously described (Manova et al., 1990). Autoradiographic detection was performed after 10 days of exposure. Slides were counterstained with H&E.

Results

Four distinct DNA elements in *Hom3a* are required for the expression of *Fgf4* in the myotomes

By creating transgenic mice containing *lacZ* constructs driven by different segments of the *Fgf4* 3'UTR and analyzing embryo whole-mounts for β -galactosidase expression, we had previously identified the conserved *Hom3a* region as the major enhancer element directing *Fgf4* expression in the myotomes (Fig. 1A). A contribution of the *Hom2* region to expression in the rostral myotomes was also observed, but transgenic constructs containing only the *Hom3a* sequences (Fig. 1A) were fully capable of expressing *lacZ* in the trunk and tail myotomes (Fraidenraich et al., 1998). Thus, to further define the *cis*-elements required for *Fgf4* expression in these structures, we concentrated our analysis on the *Hom3a* region.

As previously shown, the *Hom3a* region contains a conserved E-box (CAGCTG, nt 6356–6361) which represents the binding site for the myogenic factors MYOD and MYF5, and is required for *Fgf4* expression in the myotomes (Fraidenraich et al., 2000). However, as discussed in Introduction, transactivation by these factors appears to be necessary, but not sufficient, to drive *Fgf4* expression in the myotomes. Thus, we conducted a deletion analysis of the *Hom3a* enhancer using the same transient transgenic approach. Fig. 1B shows that a series of 5' and 3' deletions identified a minimum *Hom3a* enhancer fragment of 226 bp spanning nt 6280 to 6505 (construct 3a6, Fig. 1B) that was still capable of eliciting strong *lacZ* expression in the myotomes. The X-gal staining of myotomes driven by these mutants was observed in the trunk and tail myotomes, but not in the neck myotomes, that had been shown to require the *Hom2* region (Fig. 2) (Fraidenraich et al., 1998).

To identify more precisely the *cis*-elements in *Hom3a* relevant to myotome expression, we conducted a mutational analysis of the minimal functional *Hom3a* fragment 3a6. We mutated 12 regions within 3a6; each mutant has a 10- to 13-bp substitution and together they span the entire 3a6 sequence (Fig. 1B). All 12 mutants (Mut 1–Mut 12) were made in the context of the entire *Hom3a* sequence. Mut 1, Mut 4, and Mut 10 abolished *lacZ* expression,

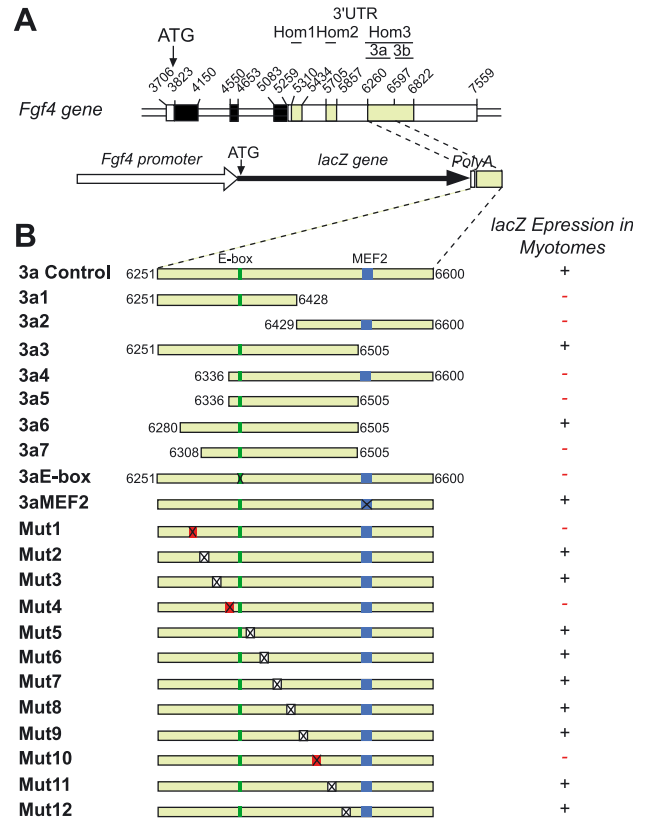


Fig. 1. (A) Structure of the murine *Fgf4* gene and *lacZ* constructs used for transgenic experiments. The *Fgf4* gene has 3 exons whose coding sequences are shown in black. In the 3'-untranslated region (3'-UTR), the three regions of high homology between mouse and human are shown in yellow rectangles (*Hom1* nt 5310–5434, *Hom2* nt 5705–5857, *Hom3* nt 6260–6822). *Hom3* is divided into 3a (nt 6260–6597) and 3b (nt 6597–6822). The *lacZ* reporter gene was placed under the control of murine *Fgf4* 5' region (nt 2443–3693), as previously described (Fraidenraich et al., 1998). (B) Seven deletion mutants of *Hom3a* region (3a1–7) and 13 nucleotide substitution mutants (3aMEF2, Mut1–12) were made and inserted to the *lacZ* construct. The construct with mutation in the E-box was previously described (Fraidenraich et al., 2000). Each of Mut1–12 mutants has a 10- to 13-bp substitution and together they span the entire 3a6 sequence. All the nucleotide substitution mutants were made within the entire *Hom3a* sequence. *lacZ* expression results obtained from each construct are listed on the right, +, *lacZ*-positive; –, *lacZ*-negative in myotomes of E11.5 embryos.

indicating that at least four distinct *cis*-acting elements, including the E-box 2, determine the activity of the *Hom3a* enhancer.

We searched the TRANSFAC database for transcription factor binding sites defined by Mut 1, Mut 4, and Mut 10, and found that the mutated region of Mut 1 contains a putative GATA factor binding site, 5'-AGATAG-3' (consensus: 5'-(A/T)GATA(A/G)-3') (Ko and Engel, 1993; Merika and Orkin, 1993). There are two potential GATA binding sites in the sequence of *Hom3a*: GATA-a (AGATAC) and GATA-b (AGATAG) (Fig. 3). Mut 1 corresponds to the GATA-b site. GATA-b completely conforms to the consensus sequence, and is well conserved among species, while the GATA-a site has one mismatch and lies outside of the

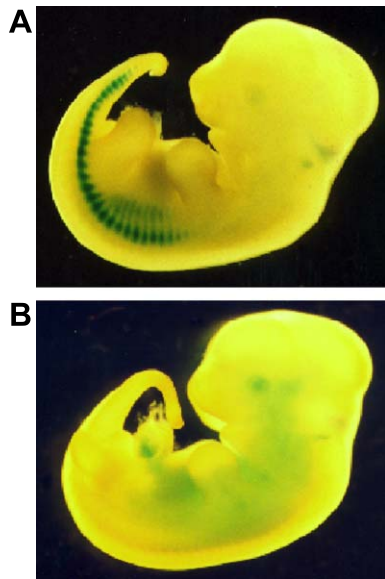


Fig. 2. Whole-mount X-gal staining of E11.5 embryos. Panel A shows an embryo that harbors the 3a6 transgene, showing the X-gal staining in the trunk and tail myotomes. Panel B shows an embryo harboring the transgene Mut1, showing no X-gal staining in the myotomes.

minimal *Hom3a* enhancer fragment defined by the 3a6 construct.

We also found that the mutated region of Mut 4 contains a putative TEF protein binding site, 5'-AAATATGCA-3'

(consensus: 5'-(A/T)(A/G)(A/G)(A/T)ATG(C/T)N-3') (Jacquemin et al., 1996). The mutated region of Mut 10 did not match any known transcription factor-binding site in the database. Interestingly, the 3' portion of *Hom3a* contains a binding site for MEF2. (Black and Olson, 1998), a myogenic transcription factor that has been shown to interact with GATA factors (Morin et al., 2000). However, mutation of this MEF2 site had no effect on *lacZ* expression in myotomes (Fig. 1B), in line with the finding that deletion of 3' sequences containing the MEF2 site (constructs 3a3, 3a6, Fig. 1) did not impair *lacZ* expression.

All the GATA factors bind to the conserved GATA binding site in Hom3a

The GATA family contains 6 factors (GATA-1–6) which are divided into 2 subfamilies, GATA-1, -2, and -3 and GATA-4, -5, and -6 (Charron and Nemer, 1999; Molkentin, 2000; Patient and McGhee, 2002). We performed EMSA to determine whether GATA factor(s) could bind to the putative GATA binding site (GATA-b). Each GATA factor was transiently expressed in transfected 293 cells. Extracts from these cells were incubated with a DNA probe containing GATA-b. As shown in Fig. 4A, all GATA factors could bind the probe. Binding was specifically competed in the presence of excess unlabelled GATA-b DNA oligonucleotide, but not in the presence of non-specific DNA oligonucleotide.

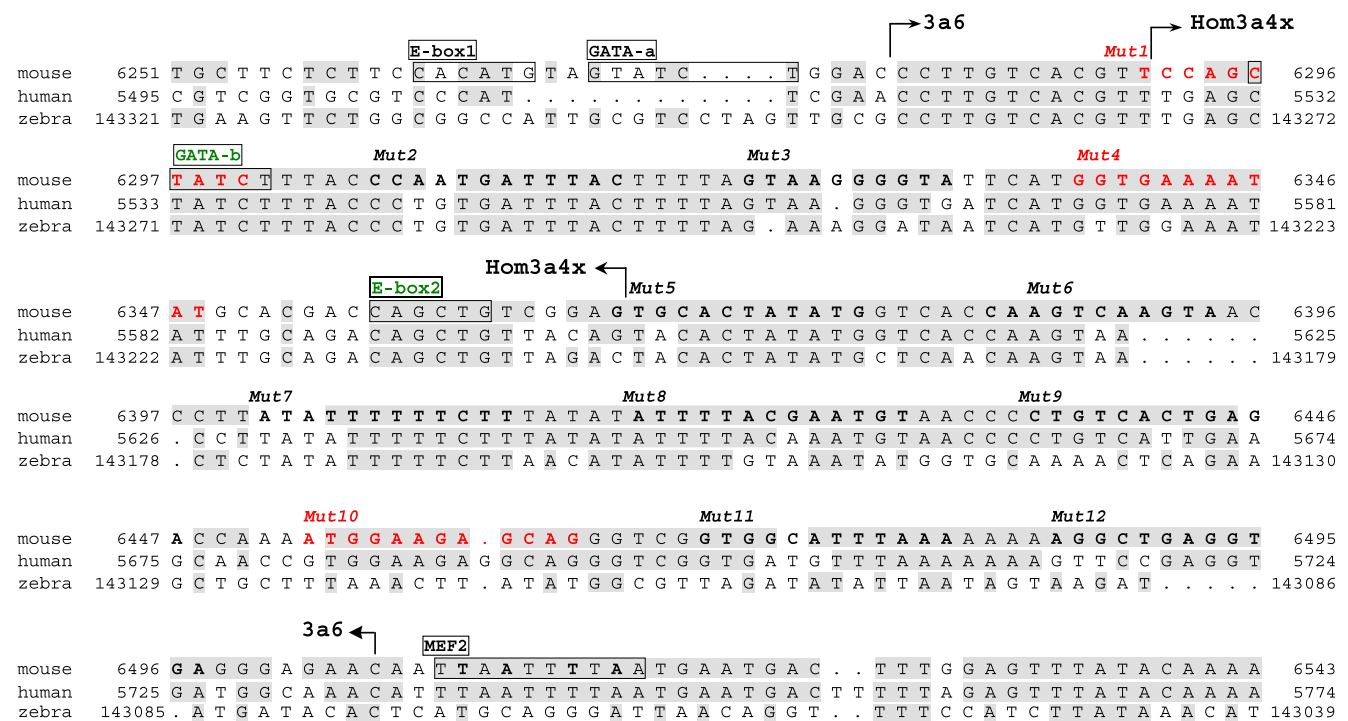


Fig. 3. Homology alignment of the *Fgf4 Hom3a* enhancer region of mouse, human, and zebrafish. Shadowed nucleotides indicate identical nucleotides to the mouse sequence. Two GATA binding sites (GATA-a, GATA-b), two E-boxes (E-box1, E-box2) and one MEF2 site are boxed. Mutated nucleotides in 3aMEF2 mutant and Mut1–12 mutants are shown in bold. Mutated nucleotides in Mut1, Mut4, and Mut 10 are shown in red. The sequences correspond to the Genbank entries X14849 (mouse), J02986 (human), and AL929586.12 (zebrafish). The nucleotide numbers of the zebrafish sequences are in reverse orientation, corresponding to the polarity of the zebrafish *Fgf4* gene (Kato, 2003).

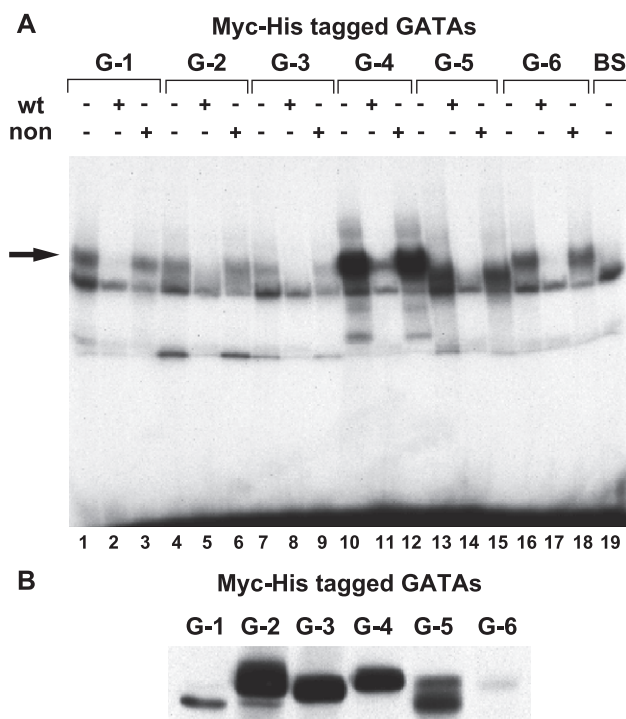


Fig. 4. All GATA factors bind to GATA-b site. (A) EMSA was performed using GATA-b probe (5'-CACGTTCCAGCTATCTTTACCCAATG - 3') with whole cell extracts derived from 293 cells which had been transfected with expression plasmids for GATA-1, lanes 1–3; GATA-2, lanes 4–6; GATA-3, lanes 7–9; GATA-4, lanes 10–12; GATA-5, lanes 13–15; GATA-6, lanes 16–18; pBluescript, lane 19. All the GATA factors contained a Myc-His tag. A 200-fold excess of cold DNAs, wt (specific) or (non-specific, 5'-GCTTCTCTCCACATGTAGTATCTGG- 3'), were used in competition assays. (B) Immunoblotting of transfected cell extracts was performed using Anti-Myc antibody.

Although all the GATA factors bound the GATA-b probe, the amount of probe bound varied significantly. Therefore, we compared the expression levels of those factors using Western analysis. Since all GATA proteins had been tagged with Myc-His peptides, proteins from transfected cell extracts were analyzed with an anti-c-Myc monoclonal antibody. Even though all the GATA factors were cloned within the same expression vector (pcDNA 3.1(-)/Myc-HisA), each factor showed a different expression level (Fig. 4B). It is possible that variations in the stability of each of the GATA proteins may be responsible for their different levels of expression. No clear correlation between the expression levels of those factors and their ability to bind the DNA probe was evident, suggesting that these closely related proteins have different affinities for the *Hom3a* GATA-b site.

GATA factors and MYOD or MYF5 synergistically activate the Fgf4 promoter

Because GATA factors have been shown to interact and synergize with other proteins to activate transcription, we investigated the possibility that GATA factors might sim-

ilarly cooperate with MYOD or MYF5. We constructed a reporter plasmid in which CAT gene expression was under the control of the minimal murine *Fgf4* promoter (Ambrossetti et al., 2000) and four copies of the *Hom3a* region spanning nucleotides 6292 to 6366. This plasmid was transfected into HeLa cells alone or together with plasmids expressing GATA factors and/or MYF5 or MYOD. While reporter gene expression was not activated by GATA proteins alone, expression of either MYOD or MYF5 alone caused a significant induction of CAT gene expression. A synergistic effect of GATA and *Myf5* or *MyoD* cotransfection was observed, but the increase in CAT expression over that produced by MYF5 or MYOD alone was only about 2-fold (data not shown). We considered the possibility that some factor expressed by HeLa cells cooperated with MYOD or MYF5 to drive gene transcription from this plasmid and masked the synergy with GATA factors. Thus, we created a new reporter plasmid in which the *Hom3a* enhancer fragment contained a small deletion of 33 nucleotides (nt 6306 to nt 6338), encompassing DNA sequences that had been shown to be dispensable for *Hom3a* activity (Figs. 1 and 3). The modified enhancer fragment still contains the GATA binding site, TEF binding site, and E-box (Fig. 5). This construct (pHom3a4xCAT) was transfected into HeLa cells together with plasmids expressing GATA factors and/or *Myf5* or *MyoD*. Fig. 5A shows that the pHom3a4xCAT plasmid had essentially no CAT activity in HeLa cells and that CAT expression was not increased by cotransfection of *Myf5*-expressing plasmids (or *MyoD*, not shown). Expression of GATA proteins 1–4 or -6 alone also did not stimulate CAT activity, but cotransfection with *Myf5* encoding plasmids resulted in a strong synergistic activation of the CAT reporter plasmid. GATA-5 increased CAT expression in the absence of *Myf5*, but this effect appeared to be non-specific, as it was produced also when we used a CAT reporter plasmid lacking the *Hom3a* sequences (Fig. 5B). The synergistic transactivation of the Hom3a4xCAT plasmid by GATA-1–4 or -6 together with *Myf5* or *MyoD* required the presence of both protein binding sites in the *Hom3a* sequences since mutation of either the GATA site or the E-box sequence abolished activation (Fig. 5C). We quantitated the synergistic activity of each GATA plasmid-*MyoD*/*Myf5* combination using the formula (Fold induction by GATA + MYF5 or MYOD) / ((Fold induction by GATA) + (Fold induction by MYF5 or MYOD)) (Fig. 5D). Among all the GATA factors, GATA-3 and GATA-4 showed the highest synergism with MYF5 or MYOD.

GATA-3 and -4 interact with MYOD and with MYF5

We wished to determine the basis for the transcriptional synergy observed between GATA factors and MYOD or MYF5. We first assessed cooperative binding of GATA proteins and MYOD or MYF5 by using a DNA probe corresponding to the sequence used to show synergistic

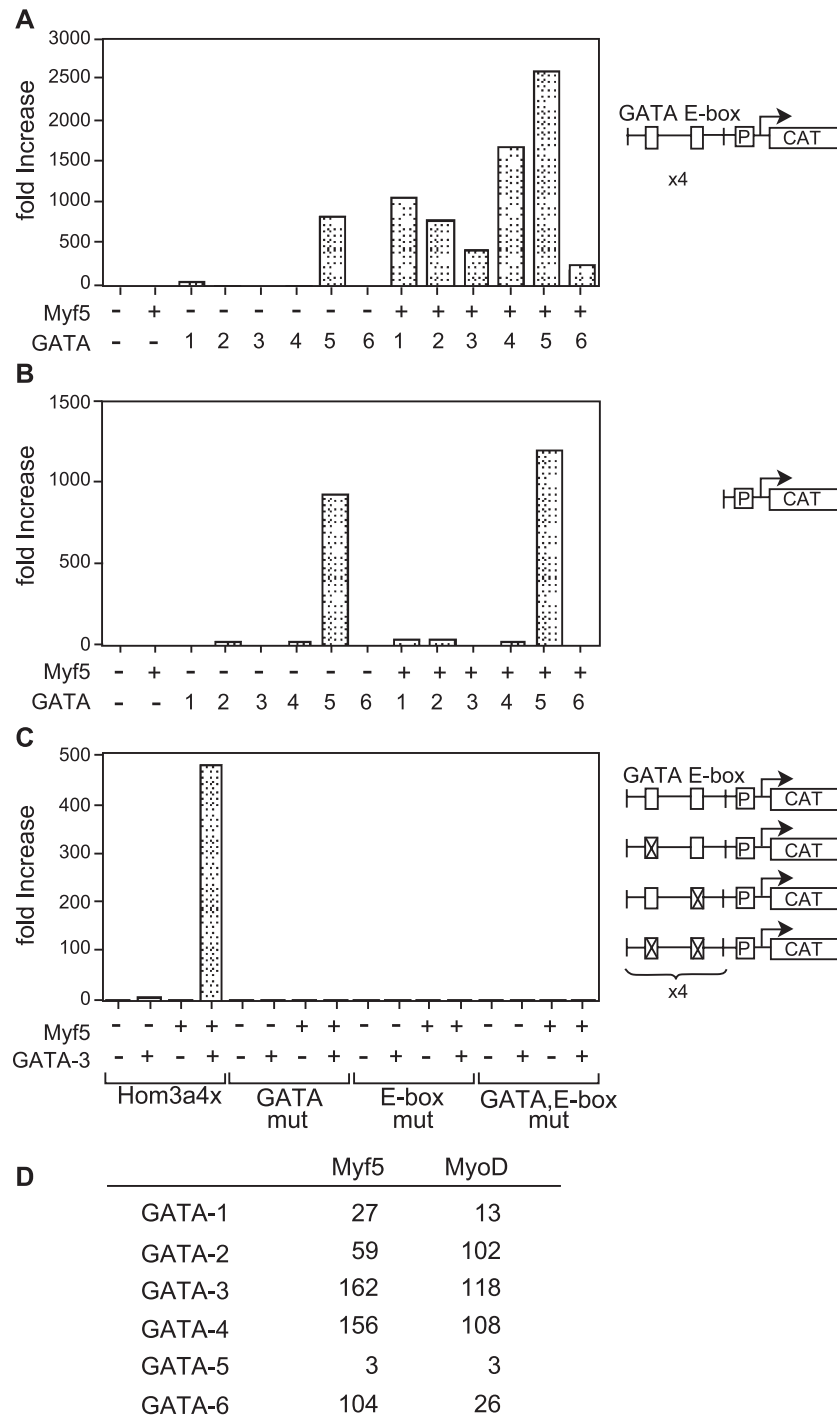


Fig. 5. GATA factors and MYF5 (or MYOD) synergistically activate the expression of a reporter plasmid driven by the *Hom3a* region. The pHom3a4xCAT reporter plasmids were transfected into HeLa cells together with CMV promoter-driven plasmids expressing GATA-1–6 proteins, MYF5 or MYOD as described in Materials and methods. A schematic representation of the pHom3a4xCAT plasmid and mutated derivatives is shown on the right. Fold increase is the level of CAT expression over that observed by transfecting the pHom3a4xCAT plasmid alone. The reporter plasmids used were (A) pHom3a4xCAT, (B) the same plasmid devoid of *Hom3a* sequences, and (C) modified pHom3a4xCAT plasmids containing base substitution mutations in the GATA binding site (AGATAG replaced by ATCGCG) or E-box (CAGCTG replaced by CCTAGG). Abbreviations: P, *Fgf4* promoter; CAT, chloramphenicol acetyl transferase.

transactivation by GATA and MYF5 proteins (4× enhancer) in EMSAs. However, we could not detect a ternary complex formed by these two proteins with the DNA probe, either because they do not bind cooperatively, or the affinity of

their interaction is low. Next, we examined whether MYF5 (or MYOD) can directly interact with GATA-3 or -4 in the absence of DNA using GST pull-down assays. We chose GATA-3 and -4 since they exhibited the highest degrees of

synergism with MYF5 or MYOD in activating the *Hom3a* reporter plasmid. Furthermore, GATA-3 has been shown to be expressed in the somites (George et al., 1994). *MyoD* or *Myf5* was expressed as GST-fusion proteins in bacteria. GATA-3 or GATA-4 was produced by in vitro translation using ^{35}S -labeled methionine. GST-MYOD or GST-MYF5, but not GST alone, was able to pull down ^{35}S -labeled GATA-3 or GATA-4 (Fig. 6B). These interactions were not affected by inclusion of up to 400 μg of ethidium bromide per ml, indicating that true protein–protein interactions were detected (Fig. 6C).

To identify the protein domains which mediate these interactions, deletion mutants or substitutional mutants of GATA-3, MYOD, and MYF5 were tested (Figs. 6D–G). GATA-3 was divided into two regions, the N-terminal region containing the N-terminal zinc finger (G3 N-ter) and the C-terminal region containing the C-terminal zinc finger (G3 C-ter). The G3 C-ter mutant bound wild type MYOD, while the G3 N-ter mutant did not (Fig. 6D). Since the zinc finger domains of GATA factors are well conserved, we produced mutants which had amino acid substitutions in the N- or C-terminal zinc fingers to determine whether they

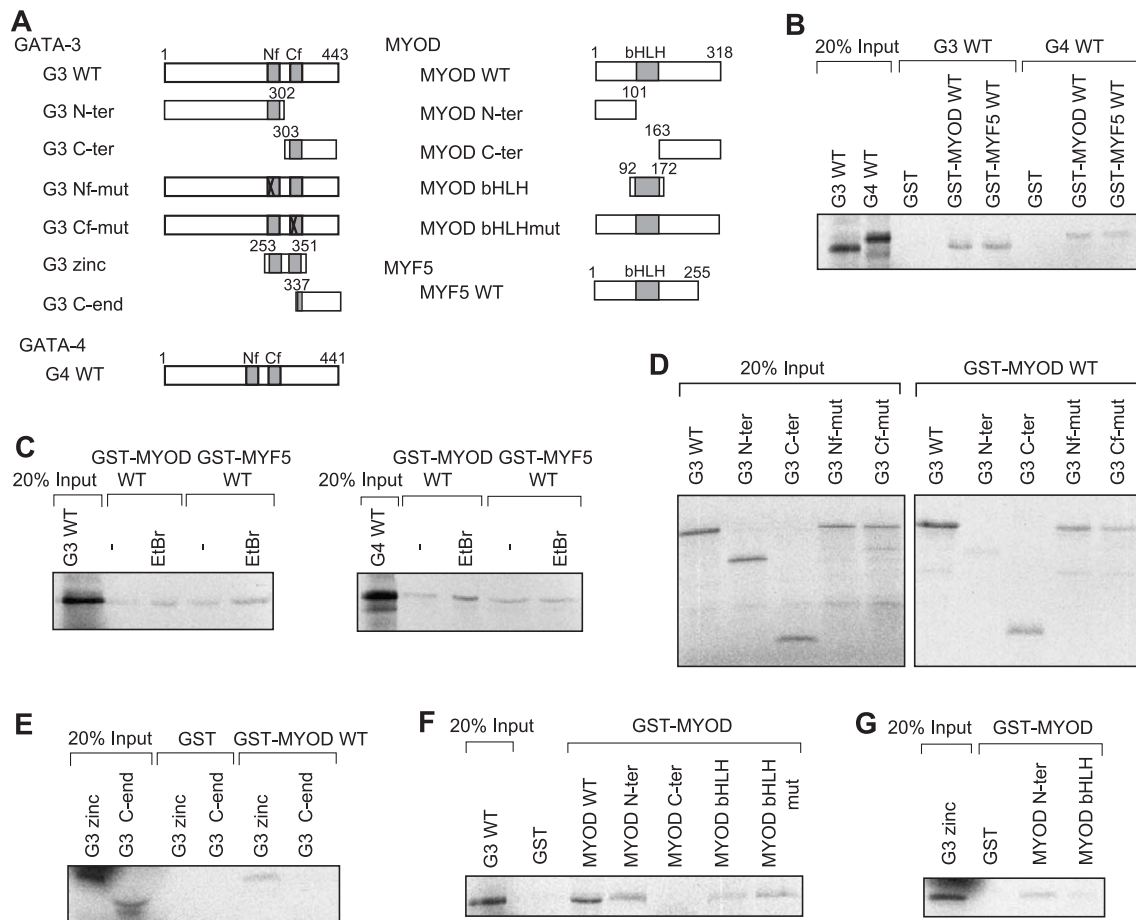


Fig. 6. GATA-3 and -4 interact with MYF5 and MYOD in the absence of DNA. (A) Constructs of GATA-3 and -4, MYOD and MYF5 used for pull-down assay. The two zinc finger domains (Nf, Cf) of GATA-3 and the bHLH domain of MYOD are shadowed. The constructs G3 Nf mut and G3 Cf mut have two alanines substituted for cysteines at aa263, 266 (Nf) and 317, 320 (Cf), respectively. MYOD bHLHmut has AAAAA substituted for KVEIL, which corresponds to the second α -helix of the bHLH structure. The regions mutated by substitution are indicated by X. (B) Pull-down assay was performed using immobilized, bacterially produced GST-fusion proteins (GST-MYOD and GST-MYF5) and ^{35}S -labeled wild type GATA-3 or GATA-4 proteins produced by in vitro translation. After incubation, the protein complexes were centrifuged, extensively washed and separated on a 10% SDS-PAGE. Bound-labeled proteins were visualized by autoradiography. (C) The samples were treated as above except that binding and wash buffers contained 400 μg of ethidium bromide per ml where indicated. (D) Mapping of GATA-3 domains required for the interaction with MYOD. GATA-3 WT, N-ter, C-ter, Nf-mut, or Cf-mut proteins were incubated with GST-MYOD WT. (E) Mapping of GATA-3 C-terminal region required for the interaction with MYOD. G3 zinc or G3 C-end were incubated with MYOD WT. (F) Mapping of MYOD domains required for the interaction with GATA-3. Full-length GATA-3 protein was incubated with full-length GST-MYOD, or GST fusion proteins containing the N terminal (N-ter), C terminal (C-ter) or bHLH domains, or mutated bHLH portions of MYOD as described in the text. MYOD c-ter did not interact with GATA-3. (G) Mapping of MYOD N-terminal and bHLH region required for the interaction with GATA-3 zinc finger domain. G3 zinc was incubated with GST fusion proteins of the MYOD N terminal (N-ter) or bHLH peptides. Abbreviations: G3, GATA-3; WT, wild type; N-ter, N-terminal domain; C-ter, C-terminal domain; Nf-mut, N-terminal zinc finger domain mutation; Cf-mut, C-terminal zinc finger domain mutation; zinc, zinc finger domain; C-end, C-terminal fragment; bHLH, bHLH domain; bHLH mut, bHLH domain mutation.

could still interact (G3 Nf-mut and G3 Cf-mut). In these constructs, two cysteins were replaced by two alanines which causes a collapse of the zinc finger structure. Interestingly, both the G3 Nf-mut and G3 Cf-mut were able to bind to wild type MYOD (Fig. 6D). Furthermore, two deletion mutants, G3 zinc and G3 C-end, were tested to map the binding site in the C-terminal region (Fig. 6E). The G3 zinc mutant bound to MYOD, but G3 C-end did not.

We then tested various MYOD mutants (Fig. 6F). MYOD was divided into three parts, N-terminal, bHLH domain, and C-terminal region. The N-terminal region and the bHLH domain bound to wild type GATA-3, but the C-terminal region did not. A five amino acid mutation in the second helix of the bHLH domain did not affect the binding to wild type GATA-3. Finally, we determined whether the GATA-3 zinc finger domain could interact with the N-terminal or bHLH regions of MYOD. Fig. 6G shows that the N-terminal region of MYOD bound to the GATA-3 zinc finger domain and that a weak interaction could also be detected with the bHLH domain. The MYF5 mutants showed similar results (data not shown). Together, these experiments demonstrate a direct protein–protein interaction between GATA-3/4 with MYOD or MYF5 in the absence of DNA. This interaction is mediated by the GATA-3/4 C-terminal zinc finger domain and by the N-terminal portion and bHLH domain of MYOD/MYF5.

Targeted deletion of Hom3a results in reduced expression of Fgf4 in the myotomes and AER, but does not cause a muscle or limb phenotype

We have shown that the *Fgf4* Hom3a region drives the expression of *lacZ* in the myotomes and limb bud AER in transgenic mice. We wished to verify further the role of *Hom3a* in *Fgf4* gene expression by assessing whether a deletion of *Hom3a* in the endogenous *Fgf4* gene would abolish or reduce *Fgf4* expression in the myotomes and AER, and also to determine whether suppression of *Fgf4* expression in these structures would affect limb or muscle development. We took advantage of the existence of distinct enhancer elements within the *Fgf4* 3'UTR, where the *Hom3b* element is required for expression in the ICM of the blastocyst and *Hom3a* for expression in the myotomes and AER (Fraidenaich et al., 1998). We reasoned that mice with only a deletion of the *Hom3a* element would not display early embryonic lethality and thus allow us to assess the effect of this mutation on limb and muscle development.

The targeting vector was designed to produce a mutated *Fgf4* allele in which the *Hom3a* element (358 bp, nt 6151 to nt 6508) could be deleted after homologous recombination. *Hom3a* was replaced with the *neo^r* gene flanked by *loxP* sites (Fig. 7A). The targeting vector was electroporated into W4-ES cells, and two clones of targeted ES cells were injected into C57BL/6 blastocysts.

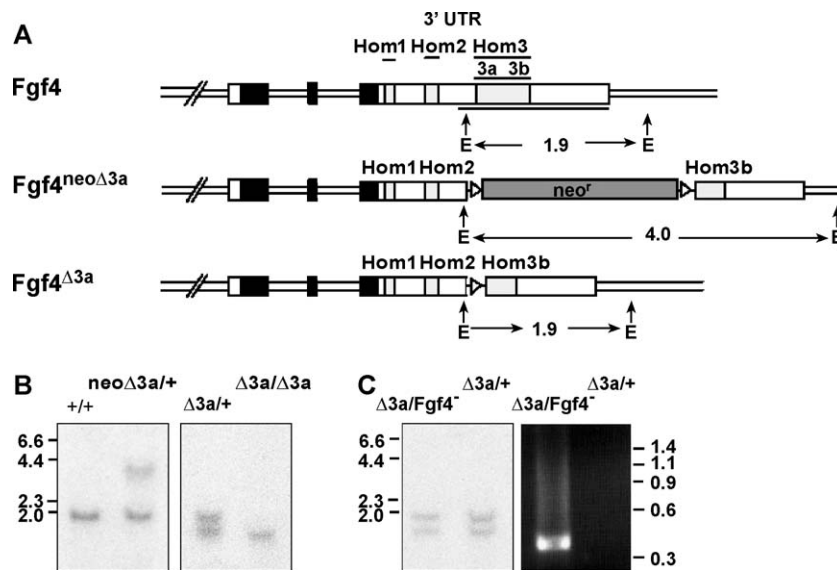


Fig. 7. (A) Strategy for construction of the *Hom3a* mutant allele. The *Fgf4* wild type allele is shown at the top (see also Fig. 1). The bar represents the DNA sequences used as a probe for southern blot analysis. E and B represent *EcoRI* and *BamHI* restriction sites, respectively. The *Fgf4^{neoΔ3a}* allele produced by gene targeting in ES cells contains a neomycin resistance cassette (*neo^r*) flanked by two *loxP* sites (triangles). Removal of the *neo^r* sequences was achieved by crossing *Fgf4^{neoΔ3a}* and CMV-*Cre* mice, resulting in the *Fgf4^{Δ3a}* allele. (B) Genotyping of wild type (+/+), heterozygotes (*neoΔ3a*/+), (*Δ3a*/+) and *Hom3a* null (*Δ3a*/Δ3a) mice by southern blot analysis. Genomic DNAs from tails were digested with *EcoRI*, and Southern blot analysis was performed with the indicated ³²P-labeled probe. Positions of DNA molecular size standards are shown to the left (kb). The wild-type allele yields a 1.9-kb DNA fragment, the *neo^r* allele yields a 4.0-kb DNA fragment, and the *Δ3a* allele a 1.7-kb DNA fragment. (C) Genotyping of *Δ3a*/*Fgf4⁻* and *Δ3a*/+ mice by Southern blot analysis (left panel) and PCR (right panel). The *Fgf4⁻* null allele contains a *neo^r* gene inserted in the first exon. Primers *NeoF*- (5'-CTCGTGCTTTACGGTATCG-3') and *Intron 1R*- (5'-GTCATCTCCTGTCTACCGGTG-3') were used for PCR to identify the *neo^r* transgene. Both genotypes show the wild type allele (1.9 kb) and *Hom3a* null allele (1.7 kb) by Southern blot analysis on the left panel, but only *Δ3a*/*Fgf4⁻* shows the *neo^r* transgene detected by PCR on the right panel. Positions of DNA size standards are shown at the left and right (kb).

To obviate possible problems due to the presence of the *neo^r* gene, male chimeras were crossed with CMV-*Cre* transgenic mice. By excising the *neo^r* gene by Cre-*loxP* recombination, heterozygotes ($\Delta 3a/+$) were born and developed normally, and we could obtain homozygotes ($\Delta 3a/\Delta 3a$) by crossing these heterozygotes (Fig. 7B). These mice appeared normal and had no detectable limb or muscle defects. Immunohistochemistry of myotome sections of E11.5 and E13.5 embryos using anti-Desmin antibodies revealed no differences in the appearance of this muscle-specific marker between wild type and $\Delta 3a/\Delta 3a$ embryos (data not shown).

We performed in situ hybridization using *Fgf4* riboprobes on sections from heterozygous ($\Delta 3a/+$) and homozygous embryos ($\Delta 3a/\Delta 3a$) at E11.5. *Fgf4* transcripts could be easily detected in both the AER and myotomes of $\Delta 3a/+$ and wild type embryos (Fig. 8 and data not shown), but were strikingly reduced, although not completely absent in $\Delta 3a/\Delta 3a$ embryos. We estimated the level of expression of *Fgf4* mRNA of $\Delta 3a/\Delta 3a$ embryos to be about 20% that of $\Delta 3a/+$ embryos, a result that would predict expression from a single $\Delta 3a$ *Fgf4* allele to be about 10% of the wild type. While this strongly reduced level of expression confirms the hypothesis that *Hom3a* is the major enhancer element responsible for *Fgf4* expression in myotomes and the AER (see Discussion), it was possible that this low level

of expression was sufficient for FGF4 to perform its function in these structures. To reduce this level of expression even further, we crossed our $\Delta 3a$ mice with heterozygous *Fgf4* knockout mice (Feldman et al., 1995) to obtain $\Delta 3a/Fgf4^{-/-}$ embryos. These animals also escaped early post-implantation lethality and did not show any apparent limb or muscle defects. Together, these experiments indicate that expression of *Fgf4* in the myotomes and AER is not essential for limb or muscle development.

Discussion

The experiments presented in this report were designed to increase our understanding of the mechanisms regulating expression of *Fgf4* in the myotomes and further determine whether *Fgf4*'s expression in these embryonic structures plays a role in muscle development. Previous results had identified a conserved 3' enhancer (*Hom3a*) in the *Fgf4* gene as the major DNA element responsible for *Fgf4* expression in the myotomes and AER, and indicated that the myotome and AER enhancers are overlapping, but distinct. Furthermore, the myogenic transcription factors MYF5 and MYOD had been shown to play a role in FGF4 expression in the myotomes by targeting a conserved E-box in *Hom3A* (Fraidenraich et al., 1998, 2000). By conducting a muta-

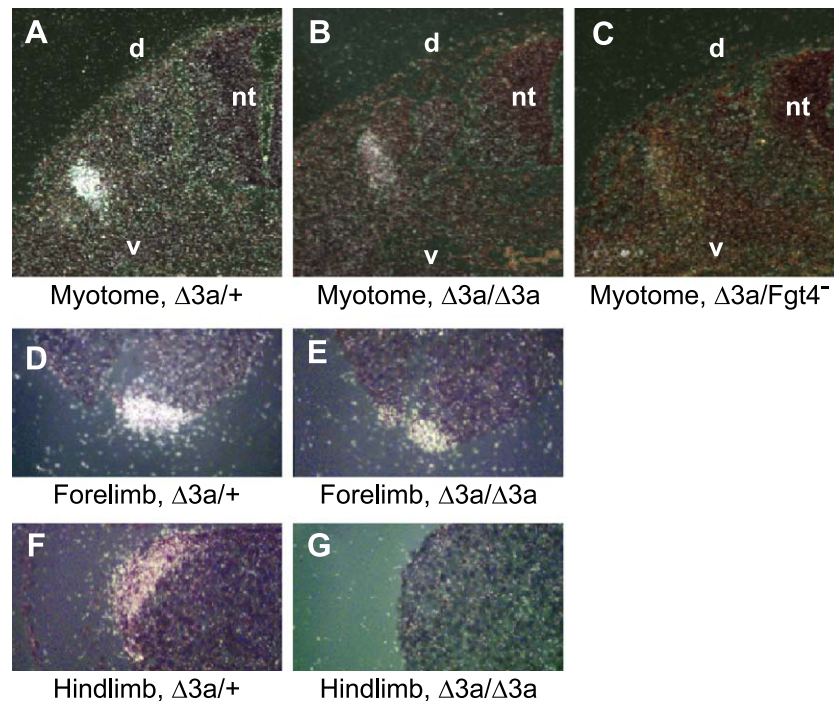


Fig. 8. *Fgf4* expression is drastically reduced in the myotomes and AER of $\Delta 3a$ embryos. In situ hybridization was performed on sections from E11.5 embryos using ^{33}P -labeled *Fgf4* antisense probes. $\Delta 3a/+$ mice were intercrossed to generate $+/+$ (wild type), $\Delta 3a/+$, and $\Delta 3a/\Delta 3a$ embryos. $\Delta 3a/+$ mice were crossed with *Fgf4* $^{+/-}$ mice to generate $\Delta 3a/Fgf4^{-/-}$ embryos. All the embryos except $\Delta 3a/Fgf4^{-/-}$ are from the same litter. The *Fgf4* RNA expression level of wild type embryos was slightly higher than that of $\Delta 3a/+$ (not shown). The experiment was performed in triplicate using different *Fgf4* probes (see Materials and methods). Numerous sections from 2 to 3 embryos for each genotype were examined. There was some variability in signal intensity from section to section in each genotype. The data shown are representative of the average signal intensity in these embryos. Analysis of tissue sections of E10.5 embryos produced similar results. Abbreviations: nt, neural tube; d, dorsal; v, ventral.

tional analysis of the ability of the *Hom3a* enhancer to drive *lacZ* expression in the myotomes of transgenic mice, we identified three additional DNA regions that are required for *Hom3a* activity. Because one of these regions contains a consensus GATA binding site that is perfectly conserved in murine, human, and zebrafish DNA, we examined the possibility that GATA factors cooperate with MYF5, or MYOD, to direct expression of *Fgf4* in the myotomes.

The GATA family of transcription factors includes six members, subdivided into two subfamilies (1–3 and 4–6) from sequence homology and expression patterns (Charron and Nemer, 1999; Molkentin, 2000; Patient and McGhee, 2002). They contain a highly conserved DNA binding domain consisting of two zinc fingers with a Cys-X₂-Cys-X₁₇-Cys-X₂-Cys motif that directs binding to the target DNA sequence (A/T)GATA(A/G). GATA-1–3 are predominantly expressed in hematopoietic stem cells, but also in other tissues. GATA-4–6 are expressed in various mesoderm- and endoderm-derived tissues, notably in the heart, where GATA-4 plays an important developmental role. Although it would have been desirable to identify all transcription factors contributing to *Fgf4* expression in the myotomes, the lack of a tissue culture system, which is equivalent to myotomes, rendered this complete analysis extremely difficult. Thus, we concentrated our efforts on the GATA factors, as these factors are known to interact and synergize with other transcription factors in promoting transcription of target genes (Anderson et al., 1998; Blokzijl et al., 2002; Dai et al., 2002; Durocher et al., 1997; Morin et al., 2000; Murakami et al., 1999; Wadman et al., 1997).

After showing that all GATA factors can bind the conserved GATA site in the *Fgf4 Hom3a* region, we tested the ability of GATA proteins to synergize with MYF5 and MYOD using a reporter plasmid containing a portion of the *Hom3a* sequence which included the conserved GATA site and E-box. GATA factors or MYF5 (or MYOD) alone had no effect on the activity of this reporter plasmid, with the exception of GATA-5, whose transactivating effect, however, appeared non-specific as it was detected also in a plasmid lacking the *Hom3a* enhancer. On the other hand, all other GATA factors showed a robust synergistic effect with MYF5 or MYOD that required both the GATA and bHLH binding sites. Thus, GATA factors can synergize with myogenic bHLH factors to promote gene expression.

To identify the mechanisms responsible for this synergism, we concentrated on GATA-3 and -4, which showed the highest degree of synergism with bHLH myogenic factors. Furthermore, GATA-3 has been shown to be expressed in the somites at E10, and its promoter can drive *lacZ* expression in the same structures at E11.5 (George et al., 1994). Although we did not detect cooperative binding of these proteins to DNA using EMSAs, we could demonstrate a direct protein–protein interaction between GATA-3 or GATA-4 with MYF5 or MYOD, using pull-down experiments and mapped the major site of contact to the C-

terminal zinc finger-containing domain of GATA-3, and the N-terminal and bHLH portion of MYOD.

GATA proteins have been shown to interact and synergize with other transcription factors to promote transcription of target genes. Interestingly, GATA-4 has been shown to interact with two other myogenic factors, MEF2 and d-Hand (Dai et al., 2002; Morin et al., 2000). The MEF2 binding site does not appear to be essential for *Fgf4* expression in the myotomes but interestingly, d-Hand is a bHLH factor, similar to MYOD and MYF5, and shares with these factors the ability to bind E-box DNA. The ability of GATA-3 and -4 to interact with MYOD maps to the same C-terminal domain, which has been shown to interact with d-Hand. However, unlike the GATA/d-Hand synergism, that between GATA proteins and MYOD/MYF5 requires that both proteins bind to their target DNA sequences. Thus, the ability of GATA proteins to interact with specific bHLH factors may be a property which is conserved in evolution.

The regulation of *Fgf4* expression in the myotomes is clearly complex since its major regulatory element, *Hom3a*, contains at least four DNA regions, which are necessary for gene expression. We have shown that both a conserved E-box and conserved GATA protein binding site play important roles in regulating the expression of the *Fgf4* gene in this embryonic structure. Since mutations in additional transcription factor binding sites abolish *Hom3a*'s ability to drive gene expression in the myotomes, MYF5/MYOD and GATA factors are probably necessary but not sufficient to promote *Fgf4* expression there and may cooperate with other factors that remain to be identified.

A further purpose of this work was to assess whether expression of *Fgf4* in the myotomes and AER was important for limb and muscle development. We reasoned that if the *Hom3a* element was the major element directing *Fgf4* expression in these structures, its deletion would selectively abolish *Fgf4* expression in the myotomes and AER. Since *Fgf4* expression in the ICM of the blastocyst depends on the *Hom3b* element (Fraidenraich et al., 1998), knockout of the *Hom3a* enhancer should not have affected blastocyst expression of *Fgf4* or early development, and thus obviate the early lethality exhibited by *Fgf4* null embryos. This strategy presents some advantages over the conditional gene knockout approach, utilizing tissue-specific Cre-*loxP*-mediated recombination, in that deletion of the enhancer element is present in the embryos from day 0, thus obviating the problem of obtaining expression of the Cre recombinase in the targeted tissues in a very precise temporal and spatial manner, as well as the possibility that the targeted gene may not be disrupted by the recombination event in every cell.

We succeeded in obtaining mice with an homozygous deletion of the *Hom3a* enhancer. Expression of *Fgf4* RNA in the myotomes and AER was strongly reduced but not totally abolished. While the *Hom3a* region had been shown to be essential for *lacZ* expression in these structures in transgenic mice, using a vector that comprised 1.2 kb of 5' sequences and more than 2 kb of sequences 3' to the coding

region (Fraidenraich et al., 1998), we could not exclude the possibility that more distant elements contribute to *Fgf4* expression in the AER and myotomes. Furthermore, we have previously observed that the *Hom2* region plays a role in *Fgf4* expression in rostral myotomes (Fraidenraich et al., 1998). The same *Hom2 Fgf4* region has recently been shown to be target for LEF-1/Wnt signaling in the tooth bud (Kratochwil et al., 2002). Wnt signaling is known to be active in the AER (Barrow et al., 2003; Capdevila and Izpisua Belmonte, 2001; Kawakami et al., 2001; Kengaku et al., 1998), and possibly the myotomes, and thus activation of gene expression through *Hom2* could be responsible for the low residual expression of *Fgf4* mRNA in the AER and myotomes of *Hom3a* mutant mice. Irrespective of the mechanisms driving residual expression of *Fgf4* mRNA in these structures, the strong reduction of *Fgf4* expression in the myotomes and AER of *Hom3a* mutant embryos confirms the hypothesis that the *Hom3a* enhancer is the major *cis*-element driving *Fgf4* expression there. These results are in contrast to a report by Moon et al. (2000), that suggested that the major regulatory elements controlling *Fgf4* expression during embryogenesis were located 5' of the coding sequences, but in line with previous results from our and other laboratories indicating that *Fgf4* expression is mainly regulated by enhancer elements in the 3' UTR (Ambrosetti et al., 1997; Curatola and Basilico, 1990; Dailey and Basilico, 2001; Kratochwil et al., 2002; Luster et al., 2000; Nowling et al., 2003).

We did not, however, observe any limb or muscle development defect in the *Hom3a* mutant mice. Since it was possible that the residual levels of expression of *Fgf4* in the myotomes and AER of these embryos could have been sufficient to fulfill its function, we sought to reduce this level of expression even further by crossing our $\Delta 3a$ mice with *Fgf4*^{+/-} mice to obtain $\Delta 3a/Fgf4$ ⁻ embryos. This should have reduced the level of expression of *Fgf4* to less than 10% of the wild type. However, again, these animals were born with no apparent limb or muscle defects. We believe it is unlikely that the almost undetectable levels of expression of *Fgf4* in the myotomes and AER of these embryos would have been sufficient to maintain FGF4 function. Indeed, other investigators have shown that transient, reduced *Fgf4* expression can lead to a limb phenotype (Sun et al., 2002). Thus, the most plausible explanation for our results is that *Fgf4* expression is not essential for limb and muscle development, probably because of functional redundancy and compensation by other FGFs expressed in these structures. Indeed, these experiments confirm the conclusions of the elegant work of Sun et al. (2002), which showed that inactivation of FGF4 alone in the AER by Cre-driven recombination had no effect on limb development, but considerably worsened the limb phenotype observed in embryos following FGF8 inactivation. It is likely that the situation in the somitic myotomes is similar. Other FGFs, notably FGF5 and FGF6, are expressed in myotomes (Han and Martin, 1993;

Haub and Goldfarb, 1991), probably providing a redundant function with FGF4. Knockout of the *Fgf5* or the *Fgf6* genes does not affect muscle development (Floss et al., 1997; Hebert et al., 1994), but inactivation of FGF4 together with FGF5 and FGF6 may reveal a function for FGF signaling in this process.

Acknowledgments

We thank Sagrario Ortega and David L. Miller for their help in the knockout experiments, Lisa Dailey and Alka Mansukhani for discussions and suggestions and Eva Deutsch for skilled technical assistance. We also acknowledge Anna Auerbach and the staff of the NYU Transgenic Mouse Facility for performing DNA microinjections. We also thank Dr. M. Yamamoto, Dr. D. Engel, Dr. E.N. Olson, Dr. S. Musco, Dr. R. Patient for the generous gift of plasmids, Dr. A. Joyner for the CMV-*Cre* mice; Dr. V. E. Papaioannou for the *Fgf4* knockout mice, and Dr. K. Manova for assistance in the in situ hybridization experiments. This investigation was supported by PHS grant CA078925 from the National Cancer Institute.

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